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Immunodominant CD4$^+$ T-Cell Epitope within Nonstructural Protein 3 in Acute Hepatitis C Virus Infection

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In acute hepatitis C virus infection, 50 to 70% of patients develop chronic disease. Considering the low rate of spontaneous viral clearance during chronic hepatitis C infection, the first few months of interaction between the patient's immune system and the viral population seem to be crucial in determining the outcome of infection. We previously reported the association between a strong and sustained CD4$^+$ T-cell response to nonstructural protein 3 (NS3) of the hepatitis C virus and a self-limited course of acute hepatitis C infection. In this study, we identify an immunodominant CD4$^+$ T-cell epitope (amino acids 1248 to 1261) that was recognized by the majority (14 of 23) of NS3-specific CD4$^+$ T-cell clones from four of five patients with acute hepatitis C infection. This epitope can be presented to CD4$^+$ T cells by HLA-DR4, -DR11, -DR12, -DR13, and -DR16. HLA-binding studies revealed a high binding affinity for 10 of 13 common HLA-DR alleles. Two additional CD4$^+$ T-cell epitopes, amino acids 1388 to 1407 and amino acids 1450 to 1469, showed a very narrow pattern of binding to individual HLA-DR alleles. Our data suggest that the NS3-specific CD4$^+$ T-cell response in acute hepatitis C infection is dominated by a single, promiscuous peptide epitope which could become a promising candidate for the development of a CD4$^+$ T-cell vaccine.

Hepatitis C virus (HCV) infection has an estimated worldwide prevalence of 0.3 to 1.5% and is a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (1, 15). More than 50% of acute infections lead to chronic disease (2), and once chronic infection is established, spontaneous recovery is exceptional. Therefore, characterization of the antiviral immune response during the first few weeks of acute hepatitis C infection in patients with self-limited disease as opposed to those developing chronic hepatitis C may allow the identification of successful antiviral immune strategies. In two recent studies of patients with acute hepatitis C infection, a strong association between a vigorous and sustained HCV-specific CD4$^+$ T-cell response and a self-limited course of acute hepatitis C infection could be demonstrated (5, 16). Although the CD4$^+$ T-cell response was directed against several HCV antigens (core, E2, nonstructural protein 3 [NS3], NS4, and NS5), in the majority of patients with self-limited disease, the response to NS3 was frequently strongest and was detected most consistently. In this study, we identify one immunodominant CD4$^+$ T-cell epitope within the NS3 protein that is recognized by the majority of patients with self-limited acute hepatitis C infection and which binds promiscuously to the most common HLA-DR alleles.

MATERIALS AND METHODS

Patients. NS3-specific CD4$^+$ T-cell clones were isolated from five patients with acute hepatitis C infection. The diagnosis was based on the following criteria: acute onset of liver disease in a previously healthy individual, absence of other viral or autoimmune hepatitis markers, and elevation of aminotransferase levels to at least five times the upper limit of normal. For all five patients, seroconversion to anti-HCV antibodies was documented.

HLA typing. Typing for HLA-DRB1 alleles was performed by using oligonucleotide hybridization with primers and oligonucleotides from the 11th International Histocompatibility Workshop (13) and a detection system using PCR and digoxigenin-11-2′-3′-dideoxy-uridinetriphosphate-labeled oligonucleotide probes (18).

HCV proteins and peptides (Fig. 1). The following fragments of HCV proteins were purchased from Microgen Inc. (Munich, Germany): core (amino acids [aa] 1 to 115), NS3 (aa 1007 to 1534), NS3-1–glutathione *S*-transferase (GST) (aa 1007 to 1278), NS3-H (aa 1207 to 1488), NS3-2–GST (aa 1271 to 1534), NS4 (aa 1616 to 1863), NS5a (aa 2003 to 2267), and NS5b (aa 2600 to 2868). cDNA derived from a genotype 1a (according to Simmonds) strain had been cloned, and the proteins were expressed in *Escherichia coli* and purified by ion-exchange chromatography followed by preparative sodium dodecyl sulfate gel electrophoresis (11). Another set of recombinant HCV proteins was obtained from Chiron, Emeryville, Calif., comprising NS3 and NS4 (c33C = aa 1192 to 1457; C100 = aa 1569 to 1931; and C200 = aa 1192 to 1931). These proteins were expressed as C-terminal fusions with human superoxide dismutase (SOD) in yeast (*Saccharomyces cerevisiae*) by methods similar to those described previously (14). All antigens were >90% pure.

Thirty-one overlapping 20-mer peptides covering aa 1207 to 1488 were synthesized by Chiron Mimotopes, Clayton, Australia, in an automatic peptide synthesizer and purified by high-pressure liquid chromatography to >90% purity. Purity and peptide identity were confirmed by mass spectrometry. Peptide aa 1248 to 1261 was synthesized at Scripps Research Institute, La Jolla, Calif.

PBMC proliferation assay. Peripheral blood mononuclear cells (PBMCs) were isolated on Ficoll-Isopaque gradients (Pharmacia, Uppsala, Sweden) and washed four times in phosphate-buffered saline (PBS). PBMCs (5×10^4/well) were incubated in 96-well U-bottom plates (Costar, Cambridge, Mass.) for 5 days in the presence of HCV proteins (1 μg/ml) in 150 μl of RPMI 1640 medium (Gibco, Grand Island, N.Y.) containing 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% human AB serum. Cultures were labeled by incubation for 16 h with 2 μCi of [^{3}H]thymidine (specific activity, 80 μCi/mmol; Amersham, Little Chalfont, United Kingdom). The cells were collected and washed on filters (Dunn, Asbach, Germany) by using a cell harvester (Skatron, Sterling, Va.), and the amount of radiolabel

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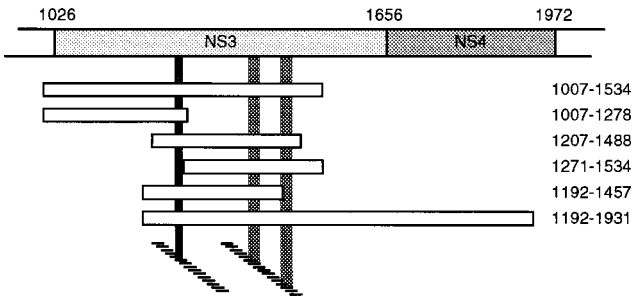

FIG. 1. Schematic representation of HCV NS3 and NS4 and the recombinant proteins and synthetic peptides used in this study. $CD4^+$ T-cell epitopes (shaded areas) and amino acid positions (numbers) are indicated.

incorporated into DNA was estimated with a beta counter (LKB/Pharmacia, Uppsala, Sweden). Triplicate cultures were assayed routinely, and the results are expressed as mean counts per minute. The stimulation index was calculated as the ratio of counts per minute obtained in the presence of antigen to that obtained without antigen. A stimulation index of >3 was considered significant.

Controls. To ensure that proliferation of PBMCs in response to HCV antigens is specific and confined to patients with HCV infection, PBMCs from 13 healthy volunteers and from patients with the following liver diseases unrelated to HCV were tested with HCV antigens: acute hepatitis B infection ($n = 8$), chronic hepatitis B infection ($n = 2$), autoimmune hepatitis ($n = 2$), and cryptogenic liver disease ($n = 2$). Proliferation assays were performed with protein concentrations from 0.1 to 10 μg/ml. Stimulation indices in all control experiments were <3. In addition, for all HCV patients, PBMCs were routinely tested with buffers that were processed in parallel to the recombinant proteins. Significant proliferation was accepted only if no proliferation in response to control buffers was observed.

Generation of T-cell clones and specificity testing. Two million PBMCs were stimulated with 1 μg of HCV protein per ml in 96-well U-bottom plates as described above. On day 6, recombinant interleukin 2 (IL-2) was added to a final concentration of 15 U/ml (kindly provided by Boehringer, Mannheim, Germany). On day 10, cells were counted and cloned at 0.5 cell/well in the presence of 3×10^4 autologous, irradiated PBMCs/well, 15 U of IL-2 per ml, and 2 μg of phytohemagglutinin (HA16; Murex Diagnostics, Dartford, United Kingdom) per ml. After 3 to 5 weeks, growing clones were tested for specificity to HCV antigens. For this, 1×10^3 to 5×10^3 clone cells were added to 3×10^4 autologous, irradiated PBMCs with and without 1 μg of HCV protein per ml and cultured for 5 days. The proliferation assay was performed as described for PBMCs.

For expansion, T-cell clones were stimulated every 3 to 5 weeks with irradiated autologous or allogeneic PBMCs, 15 U of IL-2 per ml, and 2 μg of phytohemagglutinin per ml. Earlier restimulation usually led to an unacceptable rate of cell death. Therefore, care was taken to restimulate T-cell clones only after the activation marker CD25 had returned to baseline.

FACS analyses. Triple immunofluorescence staining was performed on T-cell clones with the following combinations of conjugated antibodies: CD3 (MT301-FITC, kindly provided by E. P. Rieber, Institute for Immunology, Munich, Germany), CD4 (Leu-3a-PE; Becton Dickinson, Hamburg, Germany), CD8 (3B5-TRI-Color; Medac, Hamburg, Germany), CD25 (IL-2R1-FITC; Coulter, Hialeah, Fla.), HLA-DR (L243-PE; Becton Dickinson), and CD4 (S3,5-TRI-Color; Medac). Fluorescence-activated cell sorter (FACS) analysis was performed with a FACScan (Becton Dickinson) as described previously (9).

Lymphokine assays. NS3-specific $CD4^+$ T-cell clones were stimulated (10^5 cells/100 μl) with a combination of anti-CD2 (hybridomas 6G4 and 4B2) and anti-CD28 (hybridoma 15E8) monoclonal antibodies (1:4,000) and 1 ng of phorbol myristate acetate (Sigma, St. Louis, Mo.) per ml. Supernatants were collected after 24 h and stored at −80°C. Secretion of IL-4, IL-5, and gamma interferon (IFN-γ) was measured by using thoroughly validated in-house sandwich enzyme-linked immunosorbent assay techniques that have been described in detail elsewhere (22, 23). The sensitivities of the assays were 0.05 to 0.2 ng/ml for IL-4, 3.0 ng/ml for IL-5, and 0.1 ng/ml for IFN-γ.

Determination of HLA restriction. For determination of HLA restriction, proliferation assays were performed in the presence or absence of anti-HLA class II antibodies anti-DR (catalog no. 7730), anti-DP (catalog no. 7450), and anti-DQ (catalog no. 7360) (Becton Dickinson). Addition of 10 μl of antibody per well led to optimal inhibition of T-cell stimulation. After identification of the presenting class II molecule, fine analysis was performed using the following partially matched, homozygous, lymphoblastoid cell lines as antigen-presenting cells (APC) (12): Schu (DRA1*0102, DRB1*1501, DRB5*0101, DQA1*0102, DQB1*0602, DPA1*01, DPB1*0402), LD2B (DRA1*0102, DRB1*1501, DRB5*0101, DQA1*0102, DQB1*0602, DPA1*01, DPB1*0401), KAS011 (DRA1*0101, DRB1*1601, DRB5*02, DQA1*0102, DQB1*0502, DPA1*01/0201, DPB1*0401/1401), Boleth (DRA1*0101, DRB1*0401, DRB4*0101, DQA1*03, DQB1*0302, DPA1*01, DPB1*0401), SPO010 (DRA1*0101, DRB1*1101, DRB3*0202, DQA1*0102, DQB1*0502, DPA1*01, DPB1*02012), BM21 (DRA1*0101, DRB1*1101, DRB3*0202, DQA1*0501, DQB1*0301, DPA1*0201, DPB1*1001), BM16 (DRA1*0102, DRB1*1201, DRB3*0202, DQA1*0501, DQB1*0301, DPA1*01, DPB1*02012), CB6B (DRA1*0101, DRB1*1301, DRB3*0202, DQA1*0103, DQB1*0603, DPA1*02021, DPB1*1901), HO301 (DRA1*0102, DRB1*1302, DRB3*0301, DQA1*0102, DQB1*0605, DPA1*0201, DPB1*0501), and TEM (DRA1*0101, DRB1*1401, DRB3*0201, DQA1*0101, DQB1*05031, DPA1*01, DPB1*0401). Proliferation assays utilizing irradiated lymphoblastoid cells as APC were occasionally difficult to interpret because of high background counts, and sometimes inconsistent results were obtained even when different cell lines with identical HLA-DR alleles were utilized. To avoid these problems, we developed a FACS technique that determines activation of the $CD4^+$ T cells by their expression of CD25 after incubation with the appropriate lymphoblastoid cell line and specific antigen. Double fluorescence staining with CD4 antibodies allowed separate analysis of the $CD4^+$ T-cell clone (CD25-FITC and CD4-Tricolor). With this technique, unequivocal and highly reproducible results were obtained even when low numbers of clone cells were available. A total of 10^4 cloned T cells were incubated for 16 h with 3×10^4 lymphoblastoid cells in 96-well V-bottom plates, washed, incubated for 1 h with the fluorescent-antibody mix at 4°C, and washed again; fluorescence was measured in a FACScan (Becton Dickinson). A gate was set for $CD4^+$ cells, and binding of CD25 antibodies was expressed as median fluorescence intensity. A comparison of proliferation versus CD25 expression is shown below for two clones (see Fig. 6B and C and 6E and F). The remaining T-cell clones were routinely analyzed by CD25 expression.

HLA class II binding of HCV peptides. (i) Cells. The following Epstein-Barr virus (EBV)-transformed homozygous cell lines were used as sources of human HLA class II molecules: LG2 [DB1*0101 (DR1)]; GM3107 [DRB5*0101 (DR2w2a)]; PREISS [DRB1*0401 (DR4w4)]; BIN40 [DRB1*0404 (DR4w14)]; SWEIG [DRB1*1101 (DR5w11)]; PITOUT [DRB1*0701 (DR7)]; KT3 [DRB1*0405 (DR4w15)]; Herluf [DRB1*1201 (DR5w12)]; HO301 [DRB1*1302 (DR6w19)]; OLL [DRB1*0802 (DR8w2)]; LUY [DRB1*0803 (DR8w3)]; and HTC9074 [DRB1*1901 (DR9), supplied as a kind gift by Paul Harris, Columbia University]. In one instance, transfected L466.1 [DRB1*1501 (DR2w2b)] fibroblasts were used. Cells were maintained in vitro by culture in RPMI 1640 medium supplemented with 2 mM L-glutamine (GIBCO), 50 μM 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum (Irvine Scientific, Santa Ana, Calif.). Cells were also supplemented with 100 μg of streptomycin per ml and 100 U of penicillin per ml (Irvine Scientific). Large quantities of cells were grown in

TABLE 1. Clinical data for patients with acute hepatitis C infection

Infection type and patient no.	Sex[a]	Age (yr)	Genotype	Mode of transmission	HLA pattern	Follow-up (mo)	Peak ALT (U/liter)[b]
Acute self-limited							
1	F	36	1a	Sporadic	A2,10 B27,51 DR2,12	41	973
2	F	64	NA[c]	Sporadic	A2 B51,w60 Cw3 DR6,11	16	1,248
3	M	38	NA	Intravenous drug abuse	A1,2 B8,51 Cw7 DR2,11	22	1,466
4	F	18	1b	Transfusion	A3,11 B7,51 Cw7 DR15	12	876
Evolving chronic							
5	F	23	1b	Sexual	A2,28 B27,51 Cw2 DR2,4	16	879

[a] F, female; M, male.
[b] ALT, alanine aminotransferase. Normal values are <24 U/liter for males and <19 U/liter for females.
[c] NA, not available.

TABLE 2. Lymphokine profile of NS3-specific CD4+ T-cell clones

Patient no. and clone	Cytokine level (ng/ml)		
	IL-4	IL-5	IFN-γ
1			
1.12	<0.05	4.37	1.02
1.12a	<0.05	<3	0.89
2			
2.9	1.13	11.1	7.09
2.11	2.18	10.2	6.5
2.12	<0.2	<3	1.04
2.18[a]	<0.1	17.7	7.6
2.20	<0.2	<3	0.38
2.30[a]	5.0	12.9	3.0
2.78	<0.1	<0.2	0.42
2.79	<0.1	<0.2	0.41
3			
3.11	0.4	6.4	0.56
3.14	<0.2	<0.2	>9.0
5			
5.29	<0.1	0.75	0.24
5.34	<0.1	0.21	0.17

[a] For clones 2.18 and 2.30, fine-specificity was not determined, and these clones do not appear in Table 3.

spinner cultures. Cells were lysed at a concentration of 10^8/ml in PBS containing 1% Nonidet P-40 (NP-40) (Fluka Biochemika, Buchs, Switzerland), 1 mM phenylmethylsulfonyl fluoride (CalBioChem, La Jolla, Calif.), 5 mM sodium orthovanadate, and 25 mM iodoacetamide (Sigma Chemical). The lysates were cleared of debris and nuclei by centrifugation at 10,000 × *g* for 20 min.

(ii) Affinity purification of HLA-DR molecules. HLA class II molecules were purified by affinity chromatography as previously described (8, 20) using monoclonal antibody LB3.1 coupled to Sepharose 4B beads. Lysates were filtered through 0.8- and 0.4-μm-pore-size filters and then passed over the anti-DR column, which was then washed with 15 column volumes of 10 mM Tris in 1.0% NP-40–PBS and with 2 column volumes of PBS containing 0.4% *n*-octylglucoside. Finally, the DR was eluted with 50 mM diethylamine in 0.15 M NaCl containing 0.4% *n*-octylglucoside, pH 11.5. A 1/25 volume of 2.0 M Tris, pH 6.8, was added to the eluate to reduce the pH to ~8.0, and the eluate was then concentrated by centrifugation in Centriprep 30 concentrators at 2,000 rpm (Amicon, Beverly, Mass.).

(iii) HLA class II peptide-binding assays. Purified human class II molecules (5 to 500 nM) were incubated with various unlabeled peptide inhibitors and 1 to 10 nM ^{125}I-radiolabeled probe peptides for 48 h in PBS containing 5% dimethyl sulfoxide in the presence of a protease inhibitor cocktail. Radiolabeled probes used were HA Y307-319 (DR1), tetanus toxoid TT 830-843 (DR2w2a, DR5w11, DR7), MBP85-100Y (DR2w2b), a nonnatural peptide with the sequence YARFQSQTTLKQKT (DR4w4, DR4w14) (21), and for DR5w12, a peptide eluted from cell line C1R, EALIHQLKINPYVLS (6); there is no gene bank match. Also used as radiolabeled probes were the aforementioned nonnatural peptide for DR4 splits (DR4w15), TT 830-843 (DR8w2, DR8w3, DR9), and TT 830-843 with S836 substituted with A for DR6w19 (unpublished data). Radiolabeled peptides were iodinated by the chloramine-T method (4). Peptide inhibitors were typically tested at concentrations ranging from 120 μg/ml to 1.2 ng/ml.

TABLE 3. Summary of NS3-specific CD4+ T-cell clones and HLA restriction

Patient no. and time since onset	aa 1248–1261		aa 1388–1407		aa 1450–1469	
	Clone	HLA restriction (% anti-DR inhibition)	Clone	HLA restriction (% anti-DR inhibition)	Clone	HLA restriction (% anti-DR inhibition)
1, 5 mo	1.10	ND[a]				
	1.12	DRB1*1201 (69)				
	1.12a	DRB1*1201 (74)				
2						
1 mo	2.9	DRB1*1101 (56) DRB1*1302 (100)			2.5	ND
	2.11	DRB1*1101 (90)			2.12	DRB1*1302 (96)
	2.20	DRB1*1101 (37) DRB1*1302 (71)				
16 mo	2.78	DRB1*1101 (70)			2.65	DRB1*1302 (74)
	2.79	DRB1*1101 (50) DRB1*1302 (100)				
	2.68	DRB1*1101 (78)				
3						
1 mo	3.11	DRB1*1101 (68) DRB1*1302 (100)	3.14	DRB1*1501 (77)		
6 mo	3.110	ND	3.101	DRB1*1501 (100)		
	3.118	ND				
4, 1 mo			4.11	DRB1*1501 (98)		
			4.31	DRB1*1501 (100)		
			4.39	DRB1*1501 (100)		
			4.70	DRB1*1501 (65)		
5, 1 mo	5.29	DRB1*0401 (54) DRB1*1601 (86)				
	5.34	DRB1*0401 (65) DRB1*1601 (100)				

[a] ND, not done.

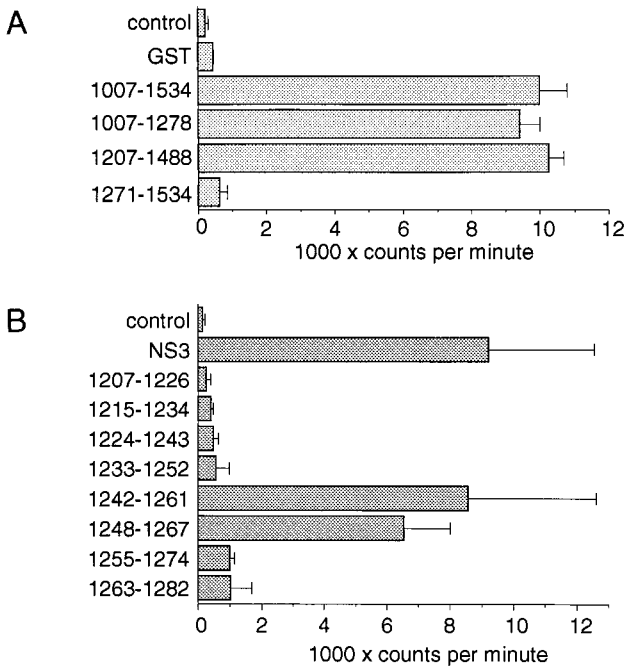

FIG. 2. (A) A representative CD4$^+$ T-cell clone from patient 3 (clone 3-11) responds to NS3 proteins aa 1007 to 1534, aa 1007 to 1278, and aa 1207 to 1488 but not to aa 1271 to 1534 or the GST control protein. (B) Testing of the T-cell clone with eight overlapping 20-mer synthetic peptides covering aa 1207 to 1282 reveals similar responsiveness to peptides aa 1242 to 1261 and aa 1248 to 1267, localizing the epitope to aa 1248 to 1261.

The data were then plotted, and the dose yielding 50% inhibition was measured. Peptides were tested in two to four completely independent experiments. The final concentrations of protease inhibitors were as follows: 1 mM phenylmethylsulfonyl fluoride, 1.3 nM 1.10-phenanthroline, 73 μM pepstatin A, 8 mM EDTA, and 200 μM *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (all protease inhibitors from CalBioChem). The final detergent concentration in the incubation mixture was 0.05% NP-40. All assays were performed at pH 7.0. Class II peptide complexes were separated from free peptide by gel filtration on TSK2000 columns, and the fraction of bound peptide was calculated as previously described (20). In preliminary experiments, the titer of the DR preparation was determined in the presence of fixed amounts of radiolabeled peptides to ascertain the concentration of class II molecules necessary to bind 10 to 20% of the total radioactivity. All subsequent inhibition and direct binding assays were then performed with these class II molecule concentrations.

RESULTS

Isolation and characterization of NS3-specific clones. NS3-specific CD4$^+$ T-cell clones were isolated from five patients with acute hepatitis C infection. The clinical data are summarized in Table 1. Four patients (no. 1 to 4) achieved spontaneous virus clearance and were HCV RNA negative as determined by PCR, with normal aminotransferase levels throughout the follow-up of 12 to 41 months. One patient (no. 5) developed chronic hepatitis C infection and remained HCV RNA positive with abnormal liver biochemistry until 5 months after disease onset, when a 6-month course of recombinant IFN-α2b was begun. This patient showed a sustained virological and biochemical response beyond 6 months after the end of treatment.

A strong NS3-specific CD4$^+$ T-cell response in the peripheral blood (mean stimulation index, 22.9; range, 5.5 to 64) was present during the first 4 weeks of acute hepatitis infection in all five patients and was maintained throughout the follow-up in the four patients with self-limited disease (mean stimulation index, 34.6; range, 14.2 to 70.3). In contrast, in the patient who did not achieve viral clearance, the NS3 response disappeared 4 weeks after disease onset and remained undetectable thereafter (mean stimulation index, 1.7; range, 1.2 to 2.8). Seven peripheral T-cell cloning experiments were performed, yielding 45 NS3-specific CD4$^+$ T-cell clones (median, six T-cell clones per cloning procedure; range, 2 to 9). For two patients, NS3-specific CD4$^+$ T-cell clones were obtained at different times, during the acute phase of disease and during follow-up (patients 2 and 3). In all cases, the cloning was performed starting with NS3-specific T-cell lines stimulated in vitro with

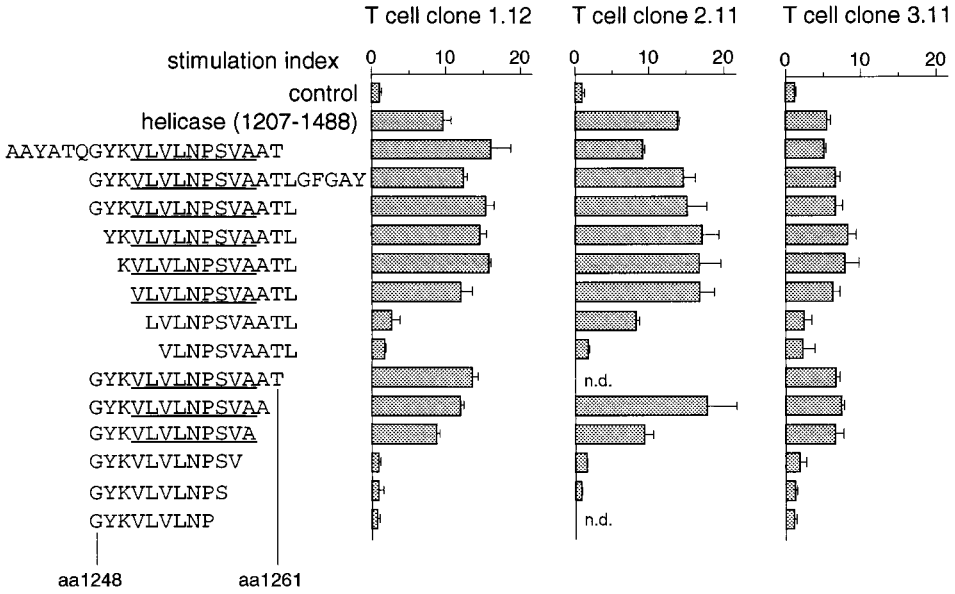

FIG. 3. Determination of the minimal epitope within peptide aa 1248 to 1261 by using a set of amino- and carboxy-terminally truncated peptides and three T-cell clones specific for aa 1248 to 1261 from three different patients. For all T-cell clones tested, aa 1251 to 1259 seems to represent the minimal epitope. However, removal of valine 1251 led to a loss of stimulation of 1.12 and 3.11 but still induced half-maximum proliferation in 2.11. n.d., not done.

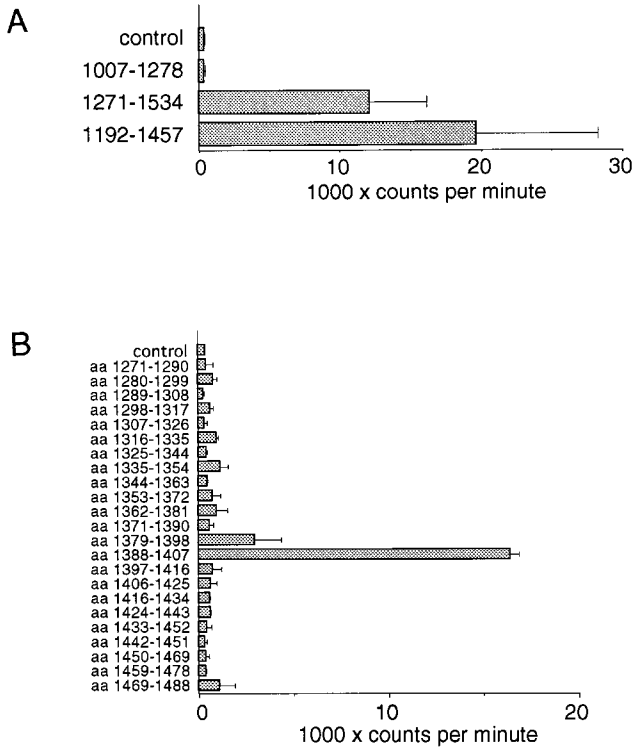

FIG. 4. (A) A representative CD4+ T-cell clone from patient 4 (clone 4-39) responds to NS3 proteins aa 1192 to 1457 and aa 1207 to 1488 but not to aa 1007 to 1278. (B) Testing of the T-cell clone with 23 overlapping 20-mer synthetic peptides covering aa 1271 to 1488 identifies aa 1388 to 1407 as the specific epitope.

our longest NS3 protein, spanning aa 1007 to 1534. Determination of the lymphokine profile revealed significant IFN-γ production by all T-cell clones; some T-cell clones also produced variable amounts of IL-4 and/or IL-5 (Table 2).

Twenty-three clones responding specifically to the aa 1007 to 1534 NS3 protein could be expanded sufficiently for further testing with shorter protein fragments and synthetic 20-mer peptides to identify their fine-specificity. The characteristics of the T-cell clones are summarized in Table 3. All six cloning procedures for patients 1, 2, 3, and 5 yielded at least one clone specific for the peptides from aa 1242 to 1261 and aa 1248 to 1267, localizing the relevant epitope to aa 1248 to 1261 (median, three T-cell clones per patient; range, two to six; Fig. 2). Fourteen of the 23 CD4+ T-cell clones, for which the fine-specificity could be determined, were specific for aa 1248 to 1261, and for two patients (1 and 5), all NS3-specific CD4+ T-cell clones responded to that epitope (Table 3). A new set of amino- and carboxy-terminally truncated peptides was synthesized, and for three clones from different patients (T-cell clones 1.12, 2.11, and 3.11), the minimal epitope was defined as aa 1251 to 1259 (Fig. 3). Whereas T-cell clones 1.12 and 3.11 were virtually identical with regard to the response to the truncated peptides, T-cell clone 2.11 seemed to depend less on aa 1251 for stimulation.

All NS3-specific CD4+ T-cell clones from patient 4, who is homozygous at the HLA-DR locus (DR15), responded to peptide 1388-1407 (Fig. 4); in addition, both cloning procedures for patient 3 yielded one CD4+ T-cell clone specific for that epitope that was also restricted by HLA-DR15. For patient 2, three NS3-specific CD4+ T-cell clones responded to a third peptide, aa 1450 to 1469 (Fig. 5).

By the use of additional recombinant protein fragments from a different source (Chiron), the epitope mapping could be confirmed: T-cell clones specific for aa 1248 to 1261 and aa 1388 to 1407 could be stimulated by proteins aa 1192 to 1457 and aa 1192 to 1931, whereas T-cell clones specific for aa 1450 to 1469 could be stimulated only by protein aa 1192 to 1931 but not protein aa 1192 to 1457, which does not contain the complete sequence (data not shown). The relevant epitopes can therefore be generated by intracellular processing of proteins of different lengths, with different fusion proteins (GST or SOD) or unfused proteins and independently of whether the proteins have been expressed in *E. coli* or yeast.

Determination of HLA restriction. In inhibition experiments using anti-HLA class II antibodies, all clones were susceptible to inhibition by anti-HLA-DR antibodies (Fig. 6A, D, G, and I; Table 3). Subsequently, the exact restriction of our HCV-specific T-cell clones was mapped by using homozygous, lymphoblastoid cell lines as APC. For clones specific for aa 1248 to 1261, the HLA-DR alleles DRB1*1101, DRB1*1201, and DRB1*0401 were identified as restriction elements (Fig. 6A to I). Presentation by DR52 and DR53 also expressed by homozygous EBV lines could be excluded on the basis of lack of presentation by EBV lines expressing similar DR52 and/or DR53 alleles but different DRB1 allelic products. When a wider panel of lymphoblastoid cell lines was used, some clones recognized the peptide also when presented by other HLA-DR molecules, irrespective of DR alleles expressed by the patient from whom the T-cell clone was isolated: a fraction of

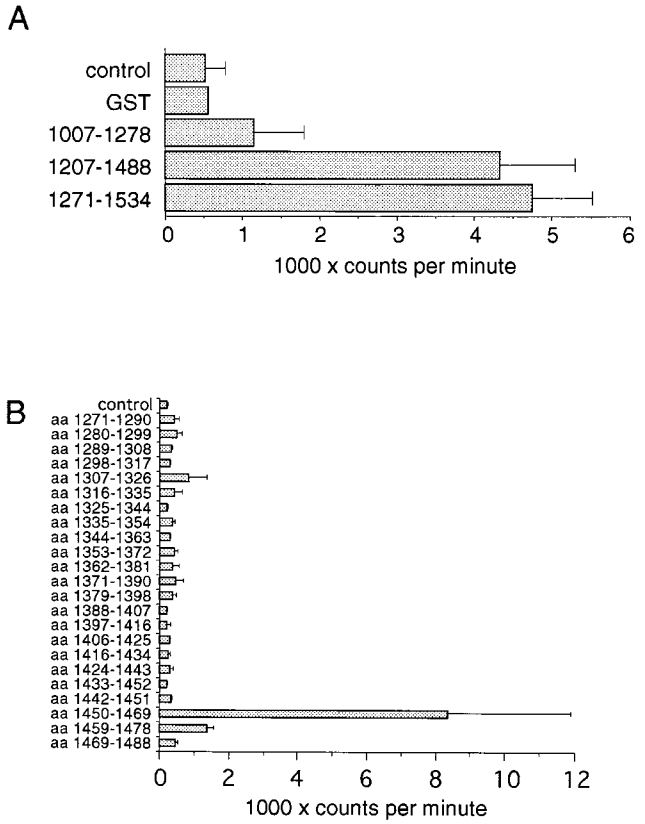

FIG. 5. (A) A CD4+ T-cell clone from patient 2 (clone 2-65) responds to NS3 proteins aa 1207 to 1488 and aa 1271 to 1534 but not to aa 1007 to 1278 or the GST control protein. (B) Testing of the T-cell clone with 23 overlapping 20-mer synthetic peptides covering aa 1271 to 1488 identifies aa 1450 to 1469 as the specific epitope.

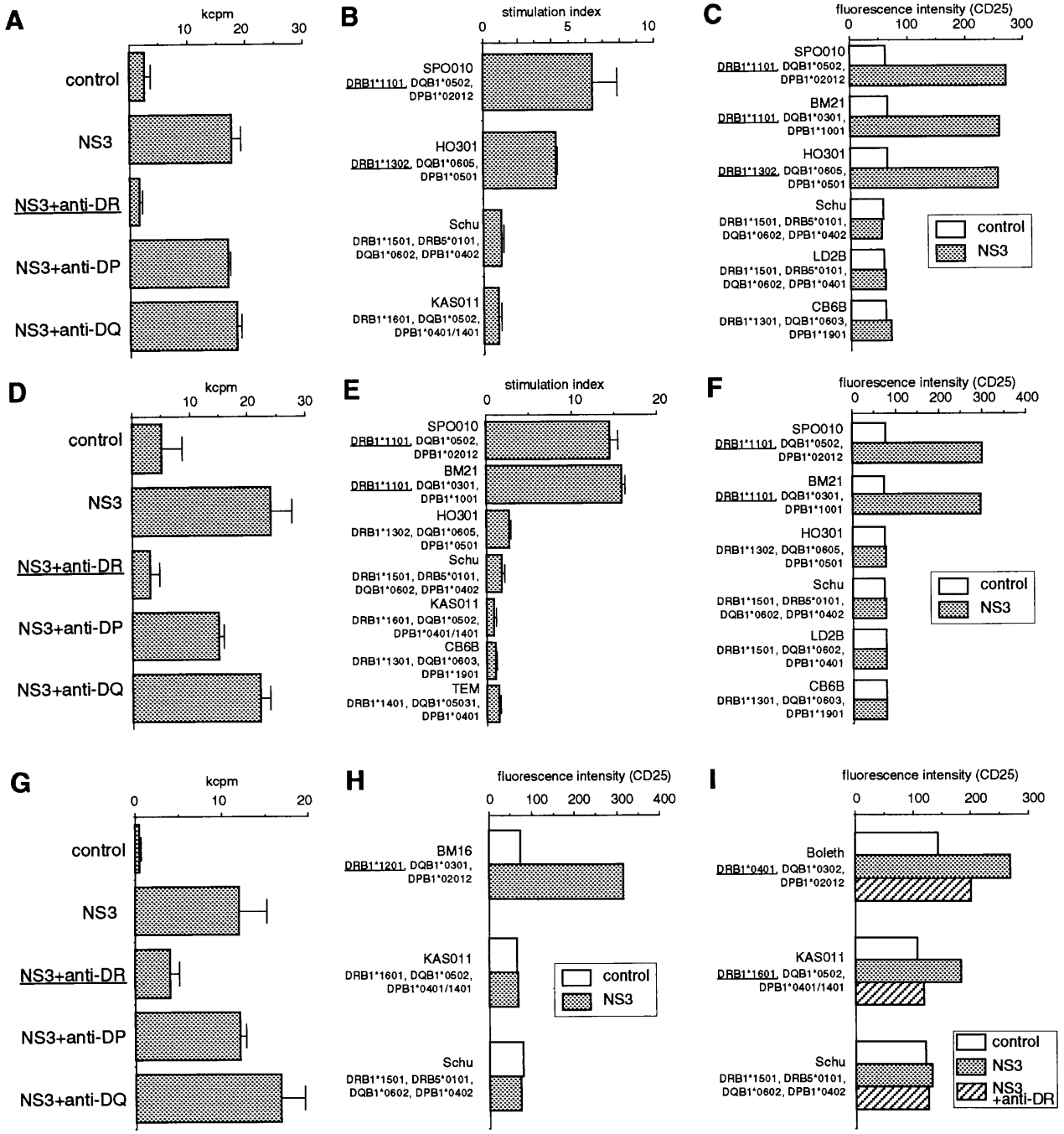

FIG. 6. HLA restriction of aa 1248 to 1261-specific $CD4^+$ T-cell clones. (A to C) Proliferation of T-cell clone 3.11 in the presence of pooled allogeneic PBMCs can be inhibited by HLA-DR antibodies but not by HLA-DP or HLA-DQ antibodies (A). Antigen-specific proliferation (B) and CD25 induction (C) occur in the presence of HLA-DRB1*1101- and HLA-DRB1*1302-positive cell lines. (D to F) Parallel experiments using clone 2.11 show restriction by HLA-DRB1*1101 without cross-reactivity to HLA-DRB1*1302. (G and H) T-cell clone 1.12 is inhibited by HLA-DR antibodies (G) and stimulated by an HLA-DRB1*1201-positive cell line (H). (I) T-cell clone 5.29 can be stimulated by HLA-DRB1*0401- and HLA-DRB1*1601-positive cell lines, and in each case stimulation is inhibited by HLA-DR antibodies. The restricting alleles are indicated (underlined).

DRB1*1101-restricted clones was also stimulated by the peptide presented by the DRB1*1302 allele (Fig. 6B and C), and one clone restricted by DRB1*0401 was also stimulated by DRB1*1601 (Fig. 6I). This promiscuous recognition could be inhibited by anti-HLA-DR antibodies (Table 3) and was of similar avidity, as judged by the antigen sensitivity (Fig. 7).

Clones specific for aa 1450 to 1469 were restricted by the allele DRB1*1302 (without cross-reactivity to DRB1*1101; data not shown); all T-cell clones specific for aa 1388 to 1407 (from both patients 3 and 5) were restricted by DRB1*1501/DRB5*0101 (data not shown). In this case, because of the tight linkage disequilibrium between DRB1*1501 and DRB5*0101, which are coexpressed in all EBV lines available to us, it is

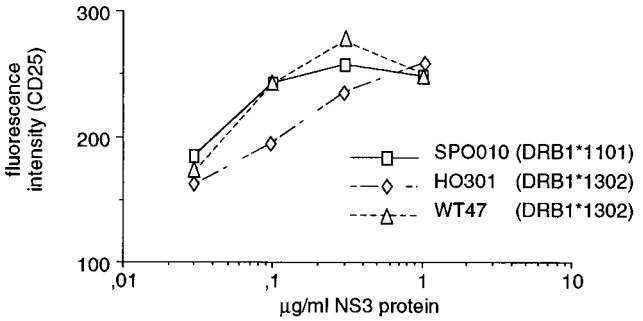

FIG. 7. Peptide aa 1248 to 1261-specific, cross-reactive T-cell clones (results for 3.11 are shown as an example) recognize their specific epitope with similar affinities if presented by HLA-DRB1*1101- or HLA-DRB1*1302-positive cell lines.

possible that either DRB1*1501 or DRB5*0101 could act as a restriction element, presenting the aa 1388 to 1407 peptide.

HLA class II affinity determination. Next, the capacities of the three epitopes described above to bind purified HLA-DR molecules in vitro were analyzed. Thirteen of the most common DR molecules, representative of more than 90% of DR types from the most common ethnic groups, were selected for this analysis. The results are shown in Table 4. It was found that the degenerate and promiscuous NS3 aa 1248 to 1261 epitope bound with high affinity (50% inhibitory concentration [IC_{50}], ≤500 nM) to 10 of the 13 molecules tested and appreciably (albeit weakly: IC_{50}, 500 to 5,000 nM) to the remaining three molecules. In particular, all DR molecules shown above to be able to present this epitope to $CD4^+$ T cells bound the NS3 aa 1248 to 1261 epitope, three of them (DRB1*1101, DRB1*1302, and DRB1*0401) with high affinity and one (DRB1*1201) with relatively weak but still significant affinity.

Synthetic peptides corresponding to the other two NS3 epitopes (aa 1388 to 1407 and 1450 to 1469) bound very selectively and with poor affinity. NS3 aa 1388 to 1407 bound its potential restricting element DRB5*0101 weakly (IC_{50}, 1,887 nM), cross-reacted marginally (IC_{50}, 17,391 nM) on DRB1*1101, and bound none of the remaining DR types tested. Similarly, NS3 aa 1450 to 1469 bound its likely restricting element DRB1*1302 only marginally (IC_{50}, 35,000 nM), cross-reacted weakly on DRB1*0701, and bound no other DR type tested.

DISCUSSION

The acute phase of hepatitis C infection, in which clearance of the virus and resolution of the disease or virus persistence and chronic disease are determined, represents the perfect situation to identify mechanisms which are considered to play a pivotal role in the interaction between virus and host. Previously, it was demonstrated that a strong and persistent HCV-specific $CD4^+$ T-cell response is associated with a self-limited course of acute hepatitis C infection (5). These data have most recently been confirmed by another group, who also demonstrated a significantly stronger HCV-specific $CD4^+$ T-cell response in patients with acute self-limited hepatitis C infection than in patients with evolving chronic hepatitis C infection (16). In the first study, NS3 seemed to be the immunodominant viral antigen for $CD4^+$ T lymphocytes, whereas the study of Missale et al. (16) found a strong $CD4^+$ T-cell response to most viral antigens, including NS3, to be associated with viral clearance. A weaker association between an HCV-specific $CD4^+$ T-cell response and viral clearance has also been described for patients with chronic hepatitis C infection who achieve a sustained response to IFN-α therapy (3, 7, 11). In those patients, however, the strongest $CD4^+$ T-cell response detected was usually to core antigen and NS4. Although $CD8^+$ cytotoxic T lymphocytes are generally thought to be the most important effector cells for the elimination of virally infected cells, in HCV infection, $CD4^+$ T cells seem to play a central role in the antiviral immune response, possibly by inducing or maintaining cytotoxic activity or by directly secreting antiviral cytokines.

$CD4^+$ T-cell responses to peptide epitopes within HCV NS4 and core antigen have previously been determined in proliferation assays using freshly isolated PBMCs (11, 16). This technique, however, may overestimate the number of $CD4^+$ T-cell epitopes; in particular, weakly positive responses are difficult to interpret (4a). Moreover, no detailed analysis of HLA restriction is possible. To avoid these problems, we used NS3-specific $CD4^+$ T-cell clones which had been isolated from polyclonal T-cell lines after stimulation with recombinant antigen to ensure that T cells are stimulated only by intracellularly processed peptides. Using that approach, we identified one immunodominant 14-aa epitope (aa 1248 to 1261) that was recognized by the majority of T-cell clones from four of five patients. It could be presented to T cells by at least five different HLA-DR alleles, and binding studies showed that 10 of 13 common HLA-DR alleles are able to bind the epitope with high affinity. Further fine-mapping with three different T-cell clones defined aa 1251 to 1259 as the putative minimal epitope. Another epitope (aa 1388 to 1407) was recognized by T-cell clones from two patients, and all these clones were HLA-DRB1*1501/DRB5*0101 restricted, suggesting that aa 1388 to 1407 may be an important $CD4^+$ T-cell epitope for patients carrying HLA-DR15. In contrast to the immunodominant epitope aa 1248 to 1261, epitope aa 1388 to 1407 and the HLA-DRB1*1302-restricted epitope aa 1450 to 1469 bound only weakly to their likely restriction elements and did not exhibit broadly cross-reactive degenerate binding capacity for other DR alleles. These observations confirm earlier studies which had suggested that degenerate binding and promiscuous recognition are associated with high-affinity binding, while selective binding is associated with weak interactions (19). They also underline the influence of HLA-DR binding affinity in determining immunodominance.

It is not known which APC present NS3 epitopes to $CD4^+$ T cells in vivo, or in what form NS3 sequences are taken up by APC. Since NS3 may not be contained in the viral particle, it is conceivable that NS3 or larger fragments of the viral polyprotein are liberated from lysed infected cells and taken up by surrounding macrophages. We could demonstrate that the relevant epitope was presented to $CD4^+$ T cells after intracellular processing of various NS3 protein fragments (including a large NS3-NS4 protein) that contained the relevant sequence, irrespective of whether the proteins were expressed in *E. coli* or yeast and whether or not they were fused to SOD or GST. It can thus be anticipated that the three epitopes can also be presented by APC in vivo even though the exact form of the source antigen is unknown.

We thus observed that a strong NS3-specific $CD4^+$ T-cell response, which is associated with viral clearance in acute hepatitis C infection, is dominated by the response to a single 14-aa epitope, aa 1248 to 1261, and can be mounted by patients with a diverse HLA background. Since viral heterogeneity and the high mutational rate of HCV are generally thought to be important factors in establishing chronic infection, we searched databases for NS3 sequences. Unexpectedly, aa 1248 to 1261 were completely conserved in all 33 genotype 1a, 1b, 1c, 2a, and 2b sequences (Table 5). Only genotype 3a shows a

TABLE 4. Affinity of binding of NS3 epitopes to a set of 13 common HLA-DR alleles

NS3 peptide (aa)	Sequence	Binding capacity[a]												
		DR1 (DRB1*0101)	DR2w2 β1 (DRB1*1501)	DR2w2 β2 (DRB5*0101)	DR4w4 (DRB1*0401)	DR4w14 (DRB1*0404)	DR4w15 (DRB1*0405)	DR5w11 (DRB1*1101)	DR5w12 (DRB1*1201)	DR6w19 (DRB1*1302)	DR7 (DRB1*0701)	DR8w2 (DRB1*0802)	DR8w3 (DRB1*0803)	DR9 (DRB1*0901)
1242–1261	AAYAAQGYKVLVLNPSVAAT	**2.9**	**48**	**483**	**18**	**86**	1,234	**103**	5,258	**11**	**96**	**60**	1,994	**240**
1248–1267	GYKVLVLNPSVAATLGFGAY	**3.5**	**42**	8,154	**9.7**	**19**	1,500	**240**	9,480	**4.1**	**23**	**80**	2,454	**20**
1248–1261	GYKVLVLNPSVAAT	**1.4**	**39**	3,695	**7.8**	**33**	**141**	**75**	4,604	**3.5**	**126**	**21**	1,124	**266**
1388–1407	GRHLIFCHSKRKCDELATKL	—	—	1,887	—	—	—	17,391	—	—	—	—	—	—
1450–1469	SVIDCNTCVTQTVDFSLDPT	—	—	—	—	—	—	—	—	35,000	12,198	—	—	—

[a] Expressed as IC_{50} (nanomolar). —, $IC_{50} > 50{,}000$ nM; boldface values, $IC_{50} \leq 500$ nM.

TABLE 5. Sequences of NS3 peptide aa 1248 to 1261 published in databases[a]

Genotype	Sequence (aa 1248–1261)
1a	GYKVLVLNPSVAAT
1b	. . . .
1c	. . . .
2a	. . . .
2b	. . . .
3a	. . N . . . .
ND[b]	. . T . . . .
ND	. . . . R . . . .

[a] The putative minimal epitope, aa 1251 to 1259, is underlined, showing that the amino acid exchange of genotype 3a lies outside the minimal epitope.
[b] ND, not determined.

change at position aa 1250 from lysine to asparagine, which lies outside the putative minimal epitope aa 1251 to 1259. Two other sequences which were not genotyped displayed one amino acid exchange each, only one of which lies within the minimal epitope. This may imply that viral escape is unlikely to be an important factor in the regulation of the $CD4^+$ T-cell response to aa 1248 to 1261.

However, we cannot exclude that by using only genotype 1a proteins to determine T-cell specificity we might have missed some viral epitopes with high variability. While it is evident that the presence of a $CD4^+$ T-cell response, which in the early phase of the disease focuses on conserved epitopes, is associated with viral clearance, the absence of the described epitope-specific $CD4^+$ T-cell response in patients developing a chronic course of disease does not necessarily imply that these individuals cannot mount an immune response against HCV proteins at that early stage; instead, their T-cell response may focus on variable epitopes of the virus, thereby offering the virus a chance to evade the immune attack. The reason these patients can't respond to the conserved epitopes despite the presence of the appropriate HLA-DR alleles is unknown at present. Interestingly, in patient 5, an initial response to epitope aa 1248 to 1261 was lost during the first 4 weeks of acute hepatitis C infection, and the patient subsequently developed chronic hepatitis C infection. This observation suggests that during the course of acute hepatitis C infection, the virus-specific immune response can be downregulated to promote viral persistence. It is not known whether HCV infection leads to exhaustion of HCV-specific T cells, as suggested for certain animal models of lymphocytic choriomeningitis virus infection (17), or whether, e.g., inefficient antigen presentation in the liver induces anergy or apoptosis. Another attractive hypothesis would be that the presence of viral proteins in the bile could induce oral tolerance to HCV (10, 24), which is supported by the clinical observation that patients with severe cholestasis clear the infection more frequently. Those mechanisms may be amenable to therapeutic intervention by a peptide vaccine with or without the addition of certain cytokines. Along these lines, studies with animal models to clarify any causal relationship of the $CD4^+$ T-cell response to NS3 and other HCV antigens with viral clearance and identification of regulatory mechanisms may lead to the development of a new therapeutic strategy for both primary immunization against HCV and the treatment of chronic hepatitis C infection.

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